The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels

Long-Uong Wang, Jiann-Chu Chen*

Department of Aquaculture, College of Life and Resource Sciences, National Taiwan Ocean University, Keelung, 202, Taiwan, ROC

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Abstract

White shrimp *Litopenaeus vannamei* held in 25% seawater were injected with TSB-grown *Vibrio alginolyticus* (1 × 10^4 cfu shrimp ^-1_), and then transferred to 5, 15, 25 (control) and 35% _o_. Over 24–96 h, the mortality of *V. alginolyticus*-injected shrimp held in 5% _o_ and 15% _o_ was significantly higher than that of shrimp held in 25% _o_ and 35% _o_, and the mortality of *V. alginolyticus*-injected shrimp held in 5% _o_ was the highest. Shrimp held in 25% _o_ and then transferred to 5, 15, 25 (control) and 35% _o_ were examined for THC (total haemocyte count), phenoloxidase activity, respiratory burst, superoxide dismutase (SOD) activity, phagocytic activity and clearance efficiency to *V. alginolyticus* after 12–72 h. The THC, phenoloxidase activity, respiratory burst, SOD activity, phagocytic activity and clearance efficiency decreased significantly for the shrimp held in 5 and 15% _o_ after 12 h. It is concluded that the shrimp transferred from 25% _o_ to low salinity levels (5 and 15% _o_) had reduced immune ability and decreased resistance against *V. alginolyticus* infection.

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Keywords: *Litopenaeus vannamei*; *Vibrio alginolyticus*; Salinity; Challenge; Phenoloxidase activity; Respiratory burst; Superoxide dismutase activity; Phagocytic activity; Clearance efficiency

1. Introduction

During the past 15 years, commercial shrimp farming mainly based on tiger shrimp *Penaeus monodon* and kuruma shrimp *Marsupenaeus japonicus* has been particularly badly hit by an epidemic of viruses like monodon baculoviros virus (MBV), white spot syndrome virus (WSSV), yellow head virus (YHV) and...
infectious hypodermal and hematopoietic necrosis virus (IHHNV) [1]. Several disease outbreaks were also due to vibriosis, including *Vibrio alginolyticus*, *Vibrio damsela*, *Vibrio harveyi*, and *Vibrio parahaemolyticus* [2–5]. These diseases have been reported to be associated with increases of *Vibrio* populations of cultured pond waters [5].

In decapod crustaceans, three types of circulating haemocytes are recognised: hyaline, semi-granular and large granular cells [6]. They are involved not only in phagocytosis, an important process of eliminating microorganisms or foreign particles [7], but also in the production of melanin by the prophenoloxidase (proPO) system [8,9]. Both semi-granular and granular cells carry out the functions of the proPO system [8]. Phenoloxidase is the terminal enzyme in the proPO system and is activated by cell polysaccharides like β-1,3-glucan, lipopolysaccharide or peptidoglycan from microorganisms through pattern recognition proteins [10].

Hyaline cells carry out phagocytosis in the immunity of crustaceans. Once a pathogen enters the haemolymph, the host’s NADPH-oxidase is activated, which in turn reduces oxygen and subsequently produces several reactive oxygen species such as superoxide anion (O$_2^-$), hydroxyl radical (·OH), singlet oxygen (¹O$_2$) and hydrogen peroxide (H$_2$O$_2$). The release of superoxide anion is known as the respiratory burst, and it plays an important role in microbicidal activity [11]. In addition, the activity of superoxide dismutase, an enzyme scavenging superoxide anion, has been measured in grass shrimp *Palaemontes argentinus* [12] and white shrimp *Litopenaeus vannamei* [13,14].

White shrimp *L. vannamei*, which is naturally distributed throughout the Pacific coast from Gulf of California to northern Peru, has become the primary species currently being cultured even in several places in the East hemisphere. This species is known to inhabit wild ranges of salinity, from 1 to 20$\,$‰ to 40$\,$‰ [15]. *L. vannamei* exhibits hyper-osmotic regulation in low salinity levels, and exhibits hypo-osmotic regulation at high salinity levels with an iso-osmotic point of 718 mOsm kg$^{-1}$ (equivalent to 25$\,$‰) [16]. Best survival of juveniles is between temperatures of 20 and 30 $^\circ$C and salinities above 20$\,$‰ [17]. Farmers are likely to add freshwater to adjust salinity levels lower than these levels, because they believe the growth of shrimp in brackish water is better than in sea water.

Change in salinity may affect the immune resistance of *L. vannamei*, and lead to its susceptibility to vibriosis and mortality. Accordingly, this study was aimed at measuring (1) the effect of salinity on the susceptibility of *L. vannamei* to *V. alginolyticus*, and (2) the effect of salinity on the immune parameters of *L. vannamei*. For the latter purpose, total haemocyte counts (THC), phenoloxidase activity, respiratory burst (release of superoxide anion), superoxide dismutase (SOD) activity, phagocytic activity and clearance efficiency of shrimp to *V. alginolyticus* were examined.

2. Materials and methods

2.1. *L. vannamei*

*L. vannamei* juveniles were obtained from a commercial farm in Pingtung, Taiwan, and acclimated in the laboratory for two weeks before experimentation. Only shrimp in the intermolt stage were used for the study. The molt stage was identified by the examination of uropoda in which partial retraction of the epidermis could be distinguished [18]. For the susceptibility experiment, test and control groups of 10 shrimp in triplicate were used. For the experiments of immune parameter assays, tests were carried out in eight replicate test groups consisting of one shrimp each in 20 l PVC tanks. In all tests, the shrimp were fed twice daily with a formulated shrimp diet (Tairoun Feed Company, Taipei, Taiwan). The shrimp ranged from 11.9 to 13.2 g, averaging 12.98 ± 0.36 g (mean ± SD) with no significant size difference among the treatments. During experiments, water temperature was maintained at 27 ± 1 $^\circ$C, pH 7.8–8.2 while salinity was maintained at 25$\,$‰, and transferred to 5, 15, 25 and 35$\,$‰ for the susceptibility test and immune parameter assays.
2.2. V. alginolyticus

A known pathogen strain V. alginolyticus (CH003) isolated from diseased L. vannamei in Pingtung, Taiwan was used for the study [14]. Stocks were cultured on tryptic soy agar (TSA supplemented with 2% NaCl, Difco) for 24 h at 26 °C and transferred to 10 ml tryptic soy broth (TSB supplemented with 2% NaCl, Difco) for 24 h at 26 °C for use as a stock bacterial broth for growth test. For challenge experiments, stock cultures were centrifuged at 7155 × g for 20 min at 4 °C. The supernatant fluid was removed and the bacterial pellet was re-suspended in saline solution (0.85% NaCl) at 0.5 × 10⁶ cfu ml⁻¹ for the susceptibility test, and 1.5 × 10⁹ cfu ml⁻¹ for the phagocytic activity and clearance efficiency tests as bacterial suspension. The concentration of bacterial suspension was measured by its absorbance at 601 nm optical density, and was calculated from a standard curve based on a series of different concentrations of bacterial suspension.

2.3. Effect of salinity on the susceptibility of L. vannamei to V. alginolyticus

Challenge tests were conducted in triplicate with 10 shrimp per replicate following the methods described by Liu and Chen [14]. Into the ventral sinus of the cephalothorax of each shrimp, 20 μl of bacterial suspension (0.5 × 10⁶ cfu ml⁻¹) was injected resulting in 1 × 10⁴ cfu shrimp⁻¹. After injection, shrimp were kept in a separate 60 l glass aquarium (10 shrimp each) containing 40 l of aerated water at 5, 15, 25 (control) and 35 °C. The experiment lasted 96 h at 27 ± 1 °C. Shrimp injected with an equal volume of sterile saline solution and kept in 5, 15, 25 and 35 °C seawater served as the unchallenged controls (Table 1).

2.4. Effect of salinity on the immune parameters of L. vannamei

For haemocyte counts and enzyme activity assays, L. vannamei (12–13 g) reared in 25 °C seawater were placed in eight replicates of 201 PVC tanks (one shrimp per tank) with 5, 15, 25 and 35 °C seawater that was renewed daily for 3 days (72 h). Haemolymph was sampled individually at the beginning of the test, and at 12, 24, 48 and 72 h. Haemolymph (100 μl) was withdrawn from the ventral sinus of each shrimp into a 1 ml sterile syringe (25 gauge) containing 0.9 ml anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55, osmolality 780 mOsm kg⁻¹). A drop of the anticoagulant–haemolymph mixture was placed on a haemocytometer to measure THC using an inverted phase-contrast microscope (Leica DMIL, Leica Microsystems, Wetzlar GmbH, Germany), while the remainder was used for subsequent tests.

Table 1

<table>
<thead>
<tr>
<th>Bacterial dose (cfu shrimp⁻¹)</th>
<th>Salinity (°C)</th>
<th>Cumulative mortality (%), time after challenge (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>6  12  24  48  72  96</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0  0  0  0  0  0</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>0  0  0  0  0  0</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>6.7 ± 3.3  6.7  6.7 ± 0.3  3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3</td>
</tr>
<tr>
<td>Control</td>
<td>35</td>
<td>6.7 ± 3.3  6.7  6.7 ± 0.3  3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>0</td>
<td>0  0  0  0  0  0</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>15</td>
<td>3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3  3.3 ± 3.3</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>25</td>
<td>0  0  0  0  0  0</td>
</tr>
<tr>
<td>1 × 10⁶</td>
<td>35</td>
<td>0  0  0  0  0  0</td>
</tr>
</tbody>
</table>

Data in the challenge groups in the same column with different superscripts are significantly different (p < 0.05) among treatments. Values are mean ± S.E. (n = 30 shrimp in each case).
Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from l-dihydroxyphenylalanine (L-DOPA) based on the method of Hernández-López et al. [19]. The details of the measurement were described previously [14]. The optical density of the shrimp’s phenoloxidase activity for all test conditions was expressed as dopachrome formation in 50 µl of haemolymph.

Respiratory burst activity of haemocytes was quantified using the reduction of nitroblue tetrizolium (NBT) to formazan as a measure of superoxide anion, as described previously [14]. The optical density at 630 nm was measured using a microplate reader (Model VERSAmax, Molecular Devices, Sunnyvale, CA, USA). Respiratory burst was expressed as NBT-reduction in 10 µl of haemolymph.

Superoxide dismutase (SOD) activity was measured by its ability to inhibit superoxide radical dependent reactions using the Ransod Kit (Randox, Crumlin, UK). The details of the measurements were described previously [14]. The optical density was measured at 505 nm, 37 °C, and the rate of reaction was estimated from the absorbance readings 30 s and 3 min after adding xanthine oxidase. A reference standard SOD was supplied with the Ransod Kit. One unit of SOD was defined as the amount required to inhibit the rate of xanthine reduction by 50%. Specific activity was expressed as SOD units ml⁻¹.

To measure phagocytic activity and bacterial clearance, experimental parameters were as described above. For phagocytic activity and bacterial clearance tests, shrimp were transferred individually to 5, 15, 25 and 35 °C. After 0, 12, 24 and 72 h of transfer, shrimp were injected in the ventral sinus with 20 µl bacterial suspension (1.5 × 10⁹ cfu ml⁻¹ in 0.85% NaCl) resulting in 3 × 10⁷ cfu shrimp⁻¹. After injection, the shrimp were held in their respective test solutions for 1.5 h at 27 ± 1 °C. Then, 100 µl of haemolymph from the ventral sinus was collected, and mixed with 100 µl and 900 µl of sterile anticoagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, pH 7.55, osmolality adjusted with glucose to 780 mOsm kg⁻¹) for the measurement of phagocytic activity and clearance efficiency, respectively.

Phagocytic activity was measured following the method described by Weeks-Perkins et al. [20]. Briefly, 200 µl of the diluted haemolymph sample was fixed with 200 µl 0.1% paraformaldehyde for 30 min at 4 °C to fix the haemocytes, and then centrifuged at 800 × g (centrifuge Model 5403, Eppendorf, Hamburg, Germany) at 4 °C. The details of measurements were described previously [14]. Two hundred haemocytes were counted. Phagocytic activity, defined as percentage phagocytosis was expressed as:

\[
\text{Percentage phagocytosis} = \left\{ \frac{\text{phagocytic haemocytes}}{\text{total haemocytes}} \right\} \times 100
\]

Clearance efficiency was measured following the method of Adams [21]. The 1 ml volume of diluted haemolymph was further diluted to 100 ml with saline solution. Three 50 µl portions of this diluted haemolymph sample were spread on separate TSA plates and incubated at 27 °C for 24 h before colonies were counted using a colony counter. The number of colony of shrimp kept in 25% was expressed as the control group, and the number of colony of shrimp transferred to 5, 15, and 35% after 12, 24, 48 and 72 h was expressed as the test group. Clearance efficiency to V. alginolyticus, defined as percentage inhibition (PI) was calculated as:

\[
\text{PI} = 100 - \left\{ \frac{\text{cfu in test group}}{\text{cfu in control group}} \right\} \times 100
\]

2.5. Statistical analysis

A multiple comparison (Tukey) test was conducted to compare the significant differences among treatments using the SAS computer software (SAS Institute Inc., Cary, NC, USA). Percent data (susceptibility study) were normalised using an arcsin transformation before analysis. For statistically significant differences, it was required that \( p < 0.05 \).
3. Results

3.1. Effect of salinity on the susceptibility of L. vannamei to V. alginolyticus

All the unchallenged control shrimp held in 5\% and 25\% survived. Only one and two out of 30 shrimp were killed in the unchallenged control shrimp held in 15\% and 35\%, respectively. By contrast, the onset of mortality occurred at 6 h in the challenged shrimp held in 5\% and 15\%. Over 24–96 h, the cumulative mortality for the shrimp transferred to 5\% and 15\% was significantly higher than for the shrimp held in 25\% and 35\%. The cumulative mortality of challenged shrimp in 25\% was the lowest, whereas the cumulative mortality of challenged shrimp transferred to 5\% was the highest among four treatments after 96 h (Table 1).

3.2. Effect of salinity on the immune parameters of L. vannamei

There were no significant differences in THC for the shrimp held in 25\% at the different sampling times. The mean (±SE) THC varied from 99.2 ± 1.6 × 10^5 to 101.4 ± 3.5 × 10^5 cells ml^-1. After 12 h, the THC decreased significantly by 49\% and 21\% for the shrimp transferred to 5\% and 15\%, respectively as compared to the shrimp in 25\% (Fig. 1A).

No significant difference in phenoloxidase activity was observed among the shrimp held in 25\% at the different sampling times. Phenoloxidase activity decreased significantly for the shrimp transferred to 5\% and 15\%. After 12 h, phenoloxidase activity decreased significantly by 67\% and 33\% for the shrimp transferred to 5\% and 15\%, respectively. After 24 h, phenoloxidase activity decreased significantly by 41\% and 18\% for the shrimp transferred to 5\% and 15\%, respectively (Fig. 1B).

No significant difference in respiratory burst was observed among the shrimp held in 25\% at the different sampling times. The respiratory burst decreased significantly for the shrimp transferred to 5\% and 15\% after 12 h, whereas the respiratory burst increased significantly by 16\% for the shrimp transferred to 35\%. After 24 h, the respiratory burst decreased significantly by 35\% and 24\% for the shrimp transferred to 5 and 15\%, respectively (Fig. 2A).

No significant difference in SOD activity was observed among the shrimp kept in 25\% at different sampling times. After 12 h, the SOD activity decreased significantly by 22\% and 7\% for the shrimp transferred to 5\% and 15\%, respectively. After 24 h, the SOD activity decreased significantly by 21\% and 9\% for the shrimp transferred to 5\% and 15\%, respectively (Fig. 2B).

At time 0 h, the phagocytic activity was 28\%. After 12 h, phagocytic activity decreased significantly to 14\% and 13\% for the shrimp transferred to 5\% and 15\%, respectively as compared to the shrimp held in 25\%. After 24 h, phagocytic activity was 20, 22, 36 and 30\% for the shrimp in 5, 15, 25 and 35\%, respectively (Fig. 3A).

A similar trend was observed for the clearance efficiency against V. alginolyticus. After 12 h, clearance efficiency decreased by 14\% and 15\% for the shrimp transferred to 5\% and 15\%, respectively. After 24 h, clearance efficiency decreased by 14\% and 11\% for the shrimp transferred to 5\% and 15\%, respectively as compared to the shrimp held in 25\% (Fig. 3B).

4. Discussion

Small abalone Haliotis diversicolor supertexta was more susceptible to V. parahaemolyticus when the animals held at 30\% were transferred to 20\% and 25\% in 24 h [22]. In the present study, we found a similar phenomenon: L. vannamei was more susceptible to V. alginolyticus when the animals were transferred to 5\%
and 15‰ from 25‰ in 24 h. Exposure of *V. harveyi* to low salinity levels (10‰ and 15‰) for 12 h before use in immersion challenge tests with larvae of the tiger shrimp *P. monodon* resulted in higher rate of mortalities [23]. It is concluded that change in salinity in water can trigger disease outbreak by affecting the defence mechanism of the host: the susceptibility of *L. vannamei* to *V. alginolyticus* is enhanced by low salinity (5‰ and 15‰).

Circulating haemocytes can be affected by extrinsic factors such as temperature, pH, salinity, dissolved oxygen and ammonia in several species of decapod crustaceans [24–26]. São Paulo shrimp *Farfantepenaeus*
Paulaensis reared in 34‰ had a significantly higher THC (20% more) as compared to the shrimp reared in 22‰ and 13‰ [25]. Giant freshwater prawn *Macrobrachium rosenbergii* reared in 15‰ had a significantly higher THC (15% more) as compared to the prawns reared in freshwater [27]. The THC of *H. diversicolor supertexta* increased by 9% for the abalone transferred to 35‰, but decreased by 30% and 52% for the abalone transferred to 25‰ and 20‰, respectively from 30‰ in 24 h [22]. A similar phenomenon was observed in the present study: the THC increased by 11% for the *L. vannamei* transferred to 35‰, but decreased by 21% and 49% for the shrimp transferred to 15‰ and 5‰, respectively from 25‰ in 12 h. Further research is needed to clarify whether the change in THC results from proliferation of the cells, or movement of cells from tissues into circulation [28], or osmosis of the water between haemolymph and medium for osmotic regulation.

Fig. 2. Respiratory burst (A) and superoxide dismutase (SOD) activity (B) in the haemocytes of *L. vannamei* kept at a salinity of 25‰ at the beginning, and after 12, 24, 48 and 72 h transfer to 5, 15, 25 and 35‰. See Fig. 1 for statistical information.
The prophenoloxidase activity increased directly with salinity for the yellowleg shrimp *Farfantepenaeus californiensis* reared in salinity levels of 28, 32, 36, 40 and 44‰ [29], and for the *L. vannamei* reared in salinity levels of 5, 15, 25 and 35‰ in the present study. Cheng et al. [22] reported that the increases of both phenoloxidase activity and respiratory burst are well correlated with the number of THC for *H. diversicolor supertexta* in the range of 20–30‰. In the present study, the increases of both phenoloxidase activity and respiratory burst are also well correlated with the number of THC for *L. vannamei* in the range of 5–35‰. This fact indicated that increases of phenoloxidase activity and respiratory burst were a consequence of increases in THC, hyaline cells and granular cells. Le Moullac and Haffner [25] indicated that the amount of transcript encoding prophenoloxidase and peroxinectin decreased by 60% and 50% when blue shrimp
Litopenaeus stylirostris were exposed to ammonia at 1.5 mg l$^{-1}$ and 3.0 mg l$^{-1}$, respectively. In L. vannamei, the peroxinection cDNA has been cloned, and its transcript is significantly reduced for the shrimp when exposed to higher temperature (34 °C) [30]. In the present study, phenoloxidase activity of L. vannamei, and the phagocytic activity and clearance efficiency to V. alginolyticus decreased when the shrimp were transferred to 5\% and 15\%. Therefore, it is expected that transcript encoding prophenoloxidase and peroxinectin may reduce for L. vannamei reared in low salinity water.

H. diversicolor supertexta when transferred to 20 and 35\% decreased its release of superoxide anion as compared to the abalone reared in 30\% [22]. In the present study, it was found that L. vannamei when transferred to 5\% and 15\% decreased the release of superoxide anion and SOD activity, as compared to the shrimp reared in 25\% and 35\%. This fact indicated that the activity of NADPH-oxidase responsible for the release of superoxide anion decreased together with a decrease in the activity of superoxide dismutase (SOD) responsible for scavenging superoxide anion. Further research is needed to examine the activities of catalase and peroxidase [31] for L. vannamei reared in different salinity levels.

Phagocytosis can be affected by environmental parameters in invertebrates [7]. For example, an elevated temperature has been reported to increase phagocytosis of American oyster Crassostrea virginica [32] and freshwater prawn M. rosenbergii [33]. The phagocytic activity and clearance efficiency of M. rosenbergii against pathogen Lactococcus garvieae were significantly lower at freshwater than those at 5\% and 15\% [33]. The phagocytic activity and clearance efficiency of V. parahaulomyticus decreased for the abalone H. diversicolor supertexta transferred to 20\% and 25\% from 30\% in 24 h [22]. In the present study, it was found that phagocytic activity and clearance efficiency of V. alginolyticus decreased for the L. vannamei transferred to 5\% and 15\% from 25\% in 12 h. This correlated with increased susceptibility of L. vannamei to V. alginolyticus when the shrimp were transferred to 5\% and 15\% from 25\% in 12 h. This fact also indicated that L. vannamei cultured at an iso-osmotic salinity level exhibited higher resistance against pathogen infection by increasing its immune ability.

In conclusion, the present study documented that the susceptibility of L. vannamei to V. alginolyticus correlated with reductions in immune parameters including phenoloxidase activity, respiratory burst, SOD activity, phagocytic activity and clearance efficiency when the shrimp were transferred to low salinity level (5\% and 15\%) from 25\%.

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References

Campa-Co´rdora AI, Herna´ndez-Saaveda NY, De Philippis R, Ascencio F. Generation of superoxide anion and SOD activity in
Taiwan abalone (Vibrio parahaemolyticus) at different salinity levels. Fish & Shellfish Immunology 2004;16:295-306.


Smith VJ, So¨derha¨ll K, Hamilton M. β-1,3-glucan induced cellular defence reactions in the shore crab, Carcinus maenas. Comparative Biochemistry and Physiology A 1984;77:635-9.


Campa-Córdora AI, Hernández-Saaveda NY, De Philippis R, Ascencio F. Generation of superoxide anion and SOD activity in


Cheng W, Juang FM, Chen JC. The immune response of Taiwan abalone Haliothis diversicolor supercetexta and its susceptibility to Vibrio parahevamolyticus at different salinity levels. Fish & Shellfish Immunology 2004;16:295-306.


Pipe RK, Coles JA. Environmental contaminations in influencing immune function in marine bivalve mollusks. Fish & Shellfish Immunology 1999;5:581-95.


